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TITLE: Modulation of Invading and Resident Inflammatory Cell Activation as a Novel Way to Mitigate Spinal Cord Injury-Associated Neuropathic Pain

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The aim of our research over the past two years was to investigate the therapeutic effect of the non-psychoactive cannabinoid cannabidiol (CBD) on spinal cord injury neuropathic pain (SCI-NP) and associated inflammation. Changes in thermal and mechanical hind-paw sensitivity were determined following moderate spinal cord contusion injury (level T9-10) in female C57Bl/5 mice treated with vehicle or CBD. Additional groups were run to assess the effect of SCI and CBD treatment on microglial activation and peripheral immune cell invasion in the spinal cord. CBD treated mice showed less neuropathic pain than vehicle-treated controls; however CBD treatment failed to improve locomotor and bladder function following SCI. CBD decreased cytokine and chemokine expression and T cell invasion into the spinal cord following spinal cord injury. CBD did not decrease microglial activation or number. These results suggest that CBD's ability to attenuate recruitment of T cells into the damaged cord leads to a reduction in the development of neuropathic pain but not an improvement of motor or bladder function. More recent experiments have also shown that exposure to low dose chronic morphine exacerbates SCI neuropathic pain while exposure to higher dose chronic morphine attenuates SCI neuropathic pain. The role of T cell and microglial activation following these exposures is currently being evaluated.

15. SUBJECT TERMS

Nothing listed

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1. INTRODUCTION

The overarching goal of the research project is to investigate the ability of a non-psychoactive constituent of the Cannabis sativa plant, cannabidiol (CBD), to attenuate neuropathic pain stemming from spinal cord injury (SCI-NP). Experiments are designed to use a mouse model of spinal cord injury in conjunction with in vitro experiments to measure the effectiveness of CBD treatment in attenuating the development of thermal and mechanical sensitivity following spinal cord injury while also testing whether these positive effects are mediated in part through protective actions on microglia and/or T cells in the spinal cord. And additional aim is to further characterize the negative inflammatory impact of opioids and alcohol on SCI recovery and neuropathic pain.

2. KEYWORDS

Cannabinoid
Cannabidiol
Spinal cord injury
Neuropathic pain
Allodynia
Inflammation
Microglia
T cells
Morphine
Alcohol
IL23
IL17
BDNF

3. ACCOMPLISHMENTS

a. **Major goals of the project.** The first objective was to obtain IACUC and ACURO approval of the animal protocol (Subtasks 1A and 1B). The major goals of the project for Year 1 were 1) to establish the time course effect of CBD treatment on SCI-NP and associated inflammatory changes (Subtask 2A), 2) to determine the effect of SCI-NP and CBD on microglial phenotype (Subtasks 2B and 2C), and 3) to determine the role of T cell and IL23 activity in SCI-NP (Subtasks 2D and 2E).

- Subtask 1A proposed approval date September 2014 → Completed October 2014
- Subtask 1B proposed approval date September 2014 → Completed February 2015
- Subtask 2A proposed completion date December 2014 → 100% completed
- Subtask 2B proposed completion date February 2015 → 100% complete
- Subtask 2C proposed completion date March 2015 → Completed July 2015
- Subtask 2D proposed completion date May 2015 → Completed August 2015
- Subtask 2E proposed completion date August 2015 → Completed January 2016

The major goals of the project for year 2 (previously year 3) were 1) to determine the effect of morphine exposure alone and in combination with SCI on pro-inflammatory

molecule expression and neuropathic pain in vehicle vs CBD treated mice (Subtask 4A), 2) to determine the Effect of morphine exposure alone or in combination with SCI on M1/M2 microglial phenotype (subtask 4B), and 3) to determine the effect of morphine exposure on infiltration of CD4+ Th1 or Th17 cells following SCI (Subtask 4C).

- Subtask 4A proposed completion date February 2016 → 50% complete
- Subtask 4B proposed completion date April 2016 → 50% complete
- Subtask 4C proposed completion date March 2015 → 50 percent complete

b. **Major accomplishments.** Major activities included 1) completion of assessment of CBD effects on motor and sensory functioning and microglia and T cell activity following spinal cord injury and 2) initial characterization of low versus high dose morphine exposure on motor and sensory recovery following SCI.

- **Significant results**

1. **CBD marginally improved motor function and did not improve bladder function following spinal cord injury.** There was a statistically significant effect of CBD treatment on BMS score, with main effects of treatment [$F(1,294) = 5.485$, $p < 0.05$] and time [$F(5,294) = 244.9$, $p < 0.001$], but no significant interaction [$F(5,294) < 1.0$, ns]. Animals treated for 5 weeks were pooled with animals treated for 10 weeks since BMS was only measured for 4 weeks post-injury at which point the two treatment groups were treated identically. CBD treatment did not increase the number of mice who recovered bladder function by 4 weeks post-spinal cord injury (Figure 1B).

Figure 1

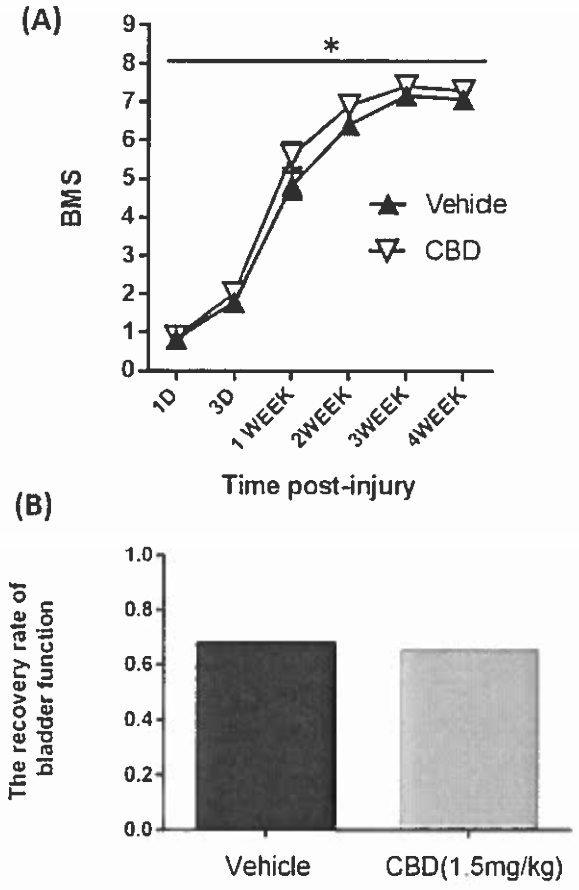


Figure 1. CBD treatment produced a modest increase in motor function (Basso mouse scale; BMS) and no improvement of bladder function in a mouse model of spinal cord injury. Because no significant differences were observed between mice in the 5 week versus 10 week treatment groups within the vehicle or CBD injection groups, 5 and 10 week treatment animals were pooled together for data analysis. There was a significant difference in BMS score (mean ± SEM) between vehicle group (n=25) and CBD treatment group (n=26) following spinal cord injury (Figure 1A). The

ratio of mice to recover bladder recovery in the vehicle group was 0.68 (n=25, 17 out of 25) and .65 in the CBD-treated group (n=26, 17 out of 26) 4 weeks after spinal cord injury (Figure 1B).

2) CBD treatment leads to less severe thermal sensitivity after spinal cord injury.
Following injury, approximately 90% of vehicle-treated mice went on to develop thermal sensitivity that progressed in severity between 4 to 10 weeks post-impact (Figure 2). Because we observed a range of the severity of thermal sensitivity, we analyzed the data by generating four sensitivity categories and calculating the percentage of animals which fell into each category for each treatment group. At week 4, approximately 12% of the mice fell into the "no change" (NC) category, 40% into the mild, 32% into the moderate and 16% into the severe sensitivity category. By week 10, 12% of the mice still had no change (similar to what has been reported previously (e.g. (Nesic, Lee et al. 2005)), 24% were in the mild category, 56% moderate, and 9% severe. Overall, CBD treatment for either 5 weeks or 10 weeks led to a leftward shift in the severity of thermal sensitivity following injury. The effects of CBD in the 5 week and 10 week treatment groups were similar (see Table 1), so data were averaged together for the two CBD-treated groups in Figure 2. At week 4 following injury, levels of sensitivity were nearly identical between the vehicle- and CBD-treated groups, while at week 10 following injury, 54% of CBD-treated mice showed mild sensitivity compared with 24% for vehicle, and only 15% of CBD-treated mice showed moderate sensitivity compared with 56% for

vehicle.

Figure 2

Thermal sensitivity

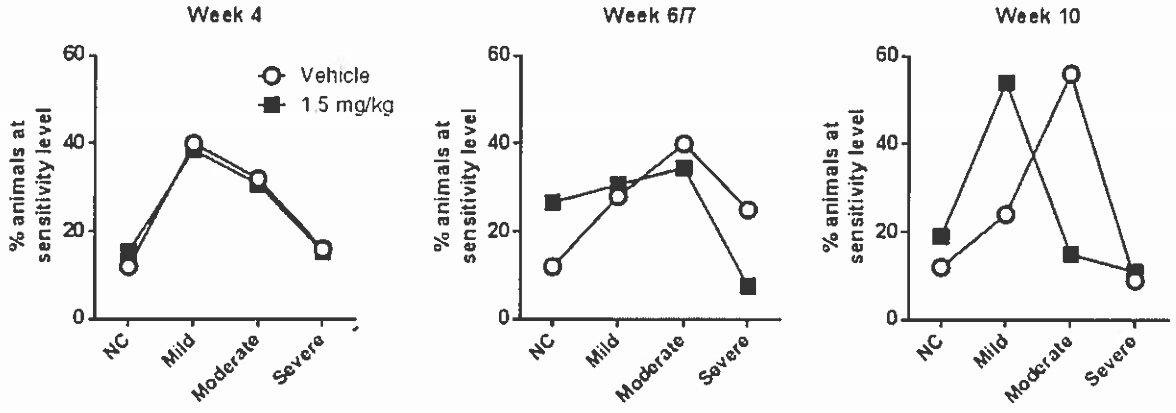


Figure 2. CBD treatment attenuates the development of thermal sensitivity in the hind paw following spinal cord injury. Because no significant differences were observed between mice in the 5 week versus 10 week treatment groups within the vehicle or CBD injection groups, 5 and 10 week treatment animals were pooled together for data analysis. The percent baseline latency scores for each mouse were used to determine the percentage of mice falling into each of four sensitivity categories: ≤33% severe, 34%-66% moderate, 67%-99% mild, ≥100% no sensitivity. There was no difference between the two groups 4 week after surgery, but by 6-7 weeks post injury there were fewer mice with severe pain in CBD-treated group as compared with the vehicle-treated group. At 10 weeks, the majority of mice in the vehicle-treated group showed moderate sensitivity, while the majority of mice in the 1.5mg/kg CBD-treated group showed mild sensitivity.

Table 1. Thermal sensitivity test in vehicle and CBD treated groups

Time after injury	Vehicle				CBD 1.5mg/kg			
4 weeks	Number of subjects		Percentage in category		Number of subjects		Percentage in category	
≥ 100%	3 out of 25		12.00%		4 out of 26		15.40%	
67%-99%	10 out of 25		40%		10 out of 26		38.50%	
34%-66%	8 out of 25		32.00%		8 out of 26		30.80%	
≤33%	4 out of 25		16.00%		4 out of 26		15.40%	
	Vehicle 5 week injection group		Vehicle 10 week injection group		CBD 1.5mg/kg 5 week injection group		CBD 1.5mg/kg 10 week injection group	
6-7 weeks	Number	Percentile	Number	Percentile	Number		Number	Percentile
≥ 100%	2 out of 18	11.10%	1 out of 7	14.30%	4 out of 18		3 out of 8	37.50%
67%-99%	6 out of 18	33.30%	1 out of 7	14.30%	5 out of 18		3 out of 8	37.50%
34%-66%	6 out of 18	33.30%	4 out of 7	57.10%	8 out of 18		1 out of 8	12.50%
≤33%	4 out of 18	22.20%	1 out of 7	14.30%	1 out of 18		1 out of 8	12.50%
10 weeks	Number	Percentile	Number	Percentile	Number	Percentile	Number	Percentile
≥ 100%	2 out of 18	11.10%	1 out of 7	14.30%	4 out of 18	22%	1 out of 8	12.50%
67%-99%	3 out of 18	16.70%	3 out of 7	42.90%	9 out of 18	50%	5 out of 8	62.50%
34%-66%	11 out of 18	61.10%	3 out of 7	42.90%	3 out of 18	16.70%	1 out of 8	12.50%
≤33%	2 out of 18	11.10%	0 out 7	0%	2 out 18	11.10%	1 out of 8	12.50%

3) **Spinal cord injury did not increase mechanical sensitivity in vehicle- or CBD-treated mice.** Unlike what was observed for thermal sensitivity, spinal cord injured mice did not develop mechanical sensitivity in either the vehicle- or CBD-treated groups (Figure 3). Two-way ANOVA revealed no main effects of treatment [$F(1,260) < 1.0$, ns], time [$F(9,260) < 1.0$, ns], and no significant interaction [$F(9,260) < 1.0$, ns].

Figure 3

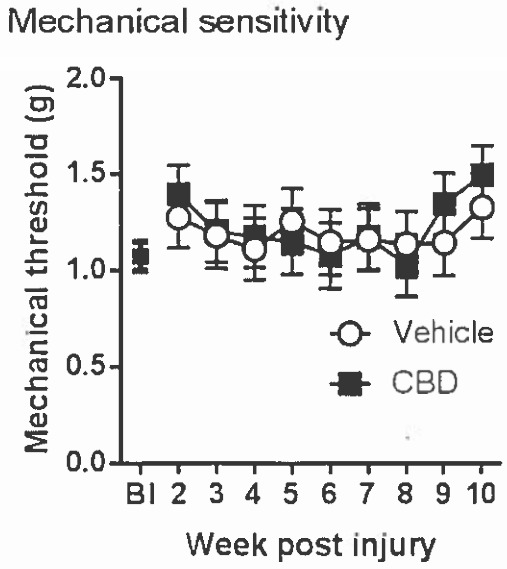
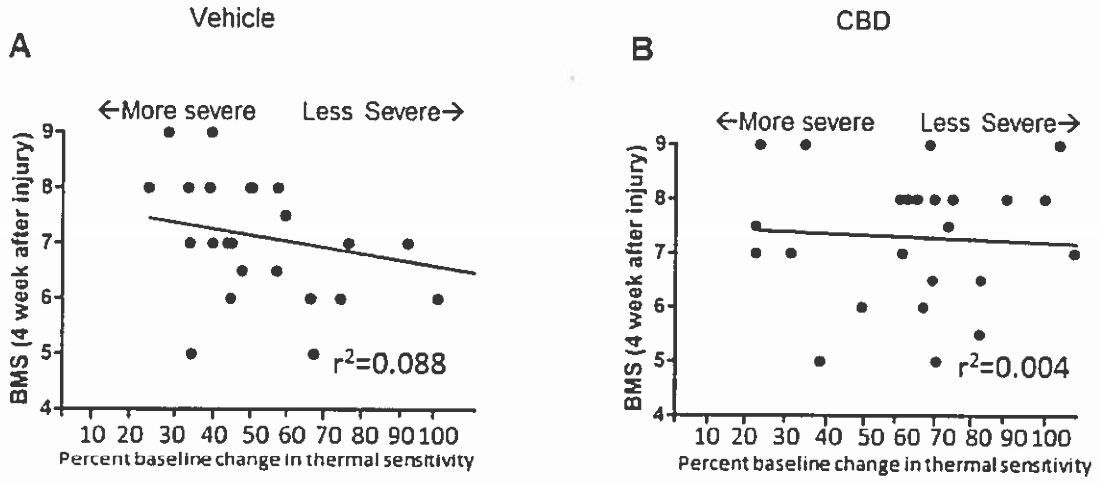


Figure 3. The present SCI mouse model was not associated with increased mechanical sensitivity. Because no significant differences were observed between mice in the 5 week versus 10 week treatment groups within the vehicle or CBD injection groups, 5 and 10 week treatment animals were pooled together for data analysis. Mechanical

sensitivity threshold did not change significantly from baseline in either the vehicle- or CBD-treated groups.

4. **Motor recovery did not correlate with later severity of thermal sensitivity in vehicle- or CBD-treated mice.** BMS sore at week 4 post-injury was not correlated with the severity of thermal sensitivity observed at week 10 in the vehicle (r^2= 0.08848) (Figure 4A) or CBD

Figure 4



treatment groups (r^2= 0.004387) (Figure4B).

Figure 4. BMS score does not correlate with severity of thermal sensitivity in spinal cord injured mice. BMS was not correlated with the severity of SCI-NP in the vehicle- (r^2= 0.088, n=25) or CBD- (r^2= 0.004, n=26) treated groups.

5. **CBD reduced cytokine expression in the injured spinal cord.** PCR array analysis of spinal cords harvested 48 hr post-injury in vehicle- and CBD-treated mice (Table 2) was performed to measure changes in gene expression of 84 key genes mediating the inflammatory response. CBD treatment was associated with the down-regulation of several chemokines and interleukins, including Ccl11, Cxcl22, Cxcl9, Xcl1, IL12b, and IL17a, and was associated with increased expression of Ccl1, Ccl12, Ccl20, Ccl24, and IL9 (Table 2). Some gene expression changes were confirmed by RT-PCR and other targets of interest were included (Figure 5). RT-PCR results showed that CBD decreased the expression of IL-23 and its receptor, IFNγ, and CXCL-9 and CXCL-11. CBD treatment did not change IL17 or IL10 expression compared to vehicle-treated injury group. There was also no effect on TRL4, Arg-1, iNOS and TNFα and IL6 expression.

Table2 Microarray analysis of gene regulation in vehicle- versus CBD-treated mice 48 hr after spinal cord injury.

Genes upregulated by CBD treatment	Fold difference	Genes down-regulated by CBD treatment	Fold difference
Ccl1	13.4064	Cxcl11	-9.6237
Ccl12	6.2994	Cxcl22	-15.7553
Ccl20	22.2876	Cxcl9	-9.2118
Ccl24	10.2979	Il2	-5.6824
Cxcl13	4.4604	Ifng	-10.152
Il5	6.3942	Il1b	-6.1986
Il9	14.2601	Il17a	-9.4012
Mstn	4.3824	Il12b	-8.4049
Tnfsf10	4.6432	Xcl1	-29.057

Figure 5

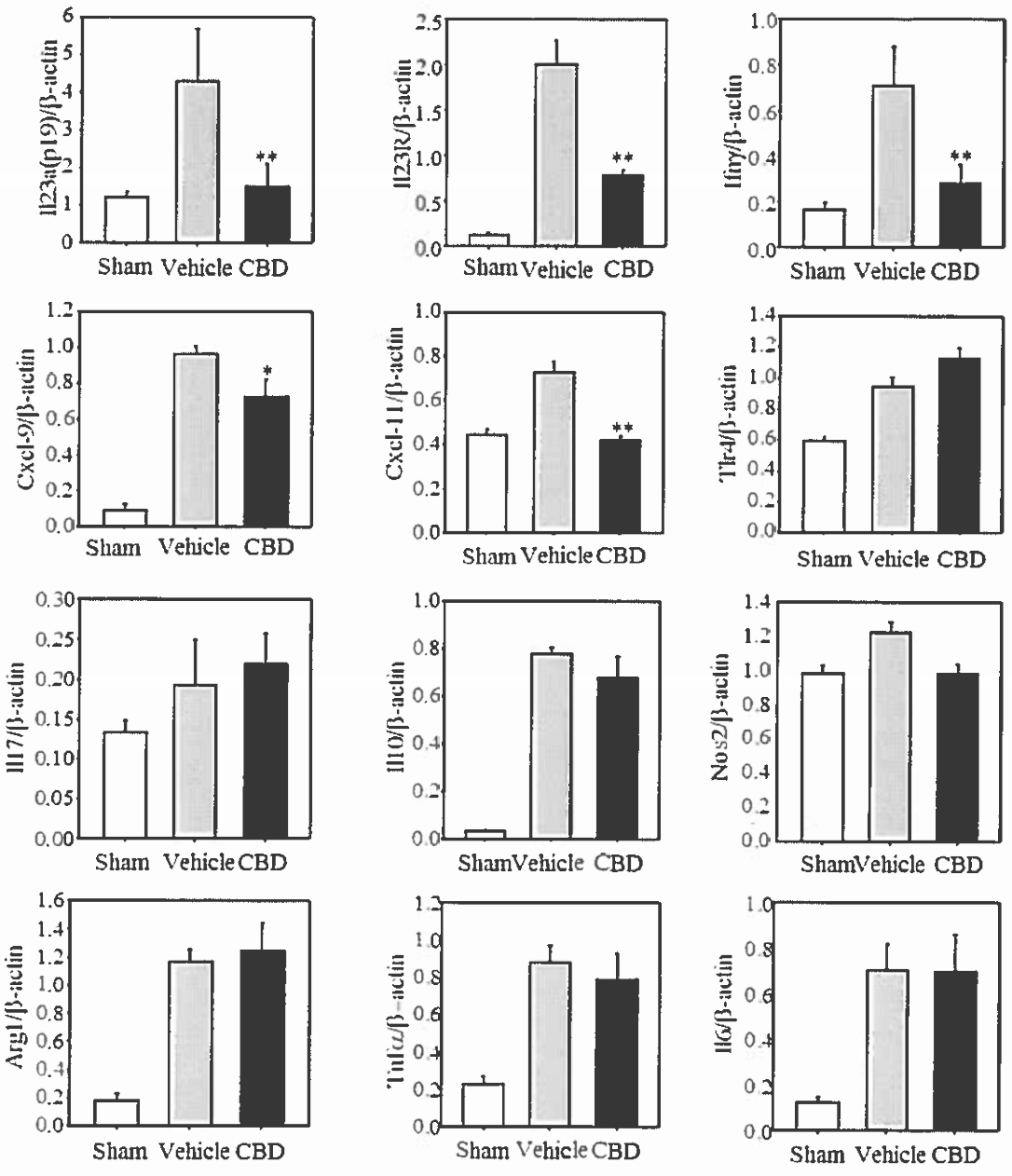


Figure 5. CBD treatment reduced inflammatory cytokine expression following spinal cord injury. RT-PCR 48 hr following spinal cord injury showed that CBD treatment reduced expression of the cytokine IL-23p19 subunit, its receptor IL-23r, the chemokines CXCL-9 and CXCL-11, and IFNγ compared with the vehicle-treated group. In contrast, there were no differences in TRL4, IL-10, Arg-1, iNOS, TNFα, and IL-6 expression *in vivo* in between the vehicle- and CBD-treated groups (n=6/group).

6) **CBD reduced CD4+ T cell numbers but not microglia number or Iba1 expression in the injured spinal cord.** Injured spinal cords of vehicle- or CBD-treated mice were harvested 2 weeks post-injury and stained antibodies for CD45, CD11b, and CD4 to measure numbers of macrophages, microglia, and T cells. CBD treatment did not decrease macrophage (CD45+high/CD11b+), microglia (CD45+intermediate/CD11b+), or total CD45+ cell populations in the spinal cord compared to vehicle-treated spinal cord-injured mice (Figure 6A). CBD treatment did significantly decrease the total number of CD4+ T cells in the injured cord compared with vehicle treatment. To ensure that the changes in cellular invasion were not the result of a systemic disease, total cell, macrophage and CD3+ T cell numbers in the spleens of CBD- and vehicle-treated animals were compared. The results showed there was no difference in cell population between the two treatment groups (Figure 6B). The negative result for microglia with flow cytometry was confirmed with immunohistochemistry using Iba1 to label microglia [$F(2,6) = 1.023$, ns]. The intensity of Iba1 immunostaining 1mm from the epicenter of injury showed no significant difference between vehicle- and CBD- treated groups (Figure 6C and D).

Figure 6

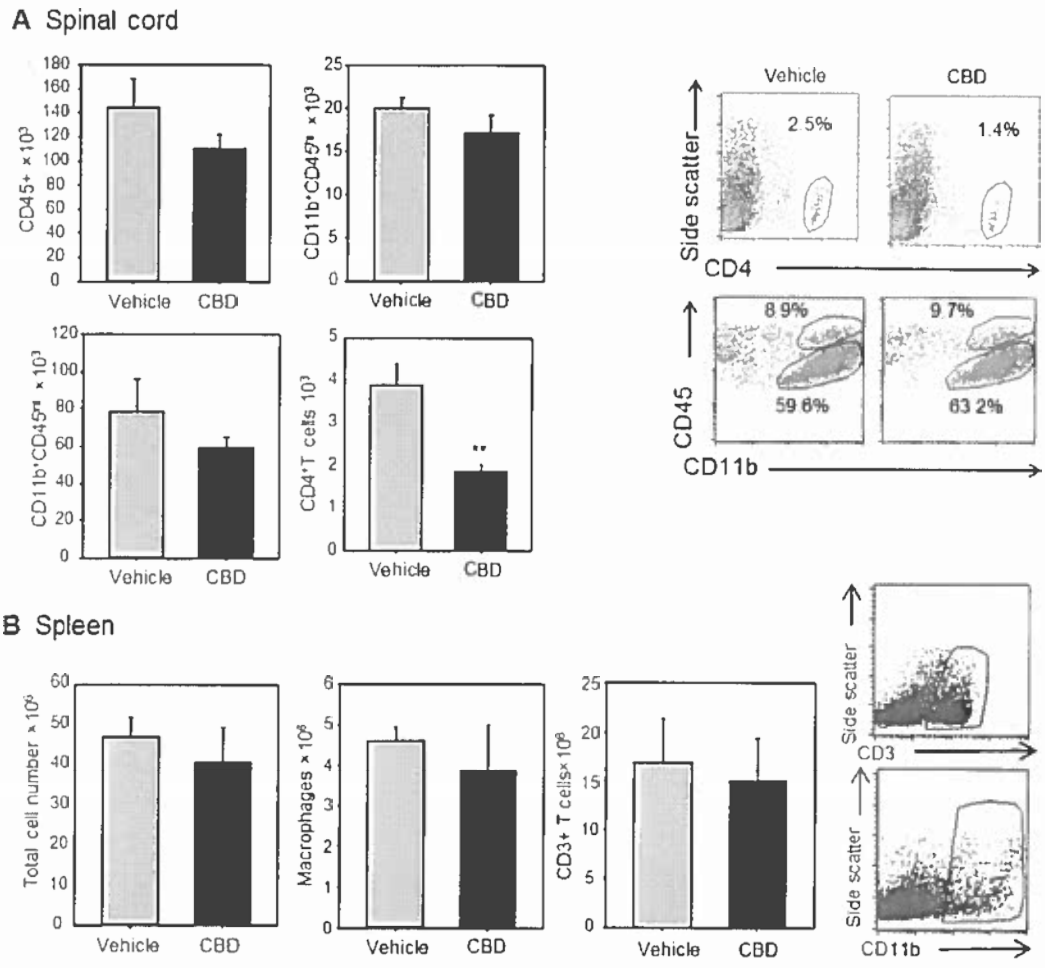

A Spinal cord
CD45+ x10³
CD11b+CD45hi x10³
CD11b+CD45hi x10³
CD4+T cells 10³
Vehicle
CBD
Vehicle
CBD
2.5%
1.4%
Side scatter
CD4
8.9%
9.7%
59.6%
63.2%
CD45
CD11b
B Spleen
Total cell number x10⁶
Macrophages x10⁶
CD3+ T cells x10⁶
Side scatter
CD3
Side scatter
CD11b
C
Sham
Vehicle
CBD
Iba-1 stain intensity
Sham
SCI
SCI+CBD

Figure 6. CBD treatment significantly decrease CD4+ T cell population in the injured spinal cord, but does not alter microglial or macrophage populations or Iba-1 expression. Spinal cords were harvested 2 weeks following SCI and vehicle or CBD treatment. CBD treatment did not decrease total CD45+ population (Figure 6A top left), microglia (bottom left) and macrophage (top right) population, but did significantly decrease CD4+ T cell population (bottom right). There were no differences in total spleen cell, T cell or macrophage numbers in the spleens of CBD-treated versus vehicle-treated animals (Figure 5B) (n=6/group). Two week-post injury spinal cords were also collected for Iba-1 staining (mean ± SEM in sham group n=2, vehicle group n=3 and CBD group n=4). Immunostained microglia in sham, vehicle-treated SCI, and CBD-treated SCI mice 1 mm from the epicenter of injury are shown at 10X magnification (Figure 5C). Consistent with flow cytometry results, the intensity of Iba-1 staining was not significantly different between the vehicle- and CBD- treated groups.

7) **The effect of CBD treatment on CGRP expression.** CGRP immunostaining in the dorsal horn of the spinal cord 1 mm from the epicenter of injury in showed that spinal cord vehicle-treated mice showed increased trend of CGRP expression as compared with sham injured animals, and that the CBD treatment group showed a trend toward attenuating that CGRP expression (Figure 7A). However, there were no significant differences in CGRP expression between sham, vehicle, and CBD groups [$F(2,6) < 1.0$, ns], (Figure 7B).

Figure 7

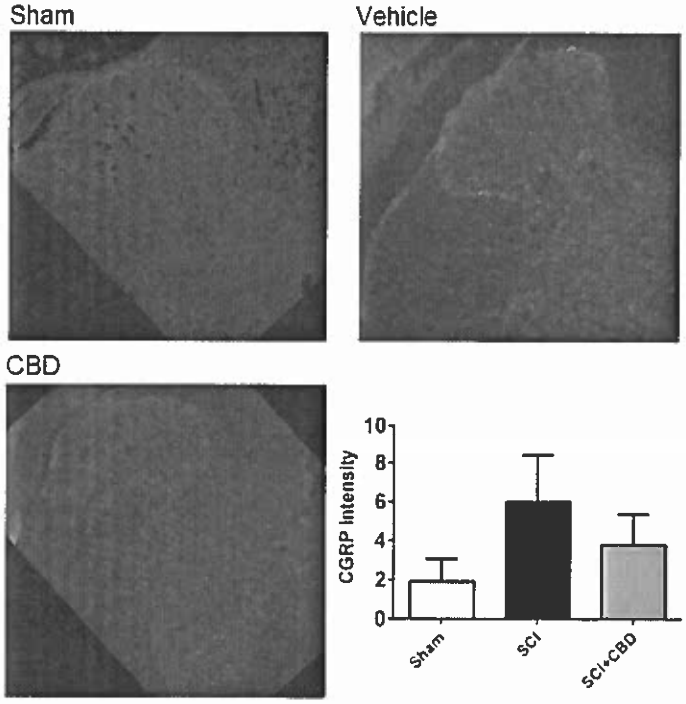


Figure 7. CBD treatment did not significantly attenuated CGRP expression in the injured spinal cord. Spinal cords were harvested 2 weeks following SCI and vehicle or CBD treatment for CGRP staining (sham group n=2, vehicle group n=3 and CBD group n=4). Immunostained CGRP in sham and 1 mm from the epicenter of injury in vehicle- and CBD- treated mice are shown at 20 x magnifications. Compared to sham-treated mice, spinal cords of injured mice show a trend for increased CGRP expression, and CBD treatment shows a trend for attenuating this effect.

8) **Effect of morphine osmotic pump exposure on SCI recovery.** Lose dose morphine exposure for 7 days following SCI led to a significant decrease in recovery of motor function, while higher dose morphine exposure for 7 days following SCI led to a significant increase in recovery of motor function (Figure 8). Low dose morphine exposure was associated with more thermal sensitivity in sham and in SCI mice, while high dose morphine was associated with less thermal sensitivity in SCI mice.

Figure 8.

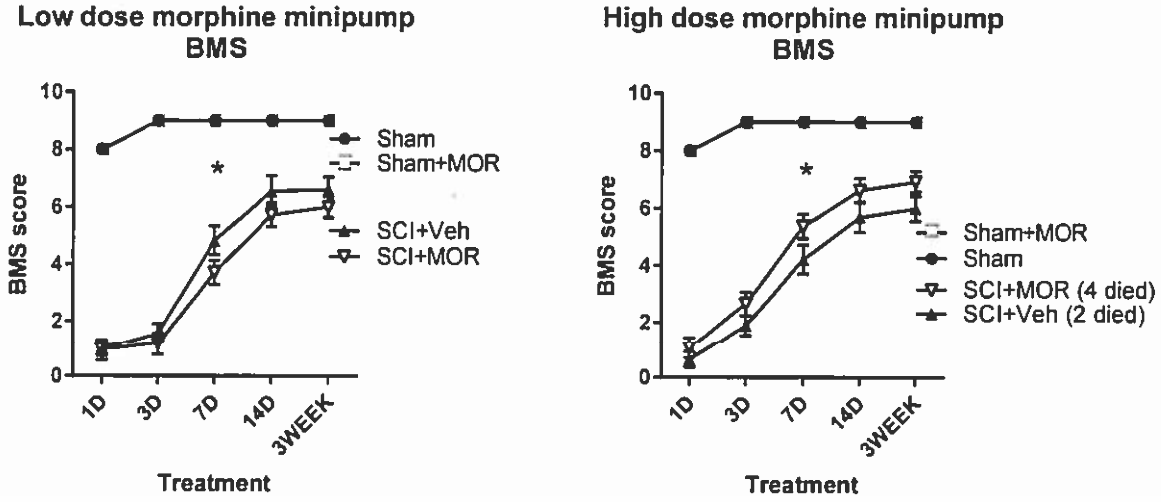
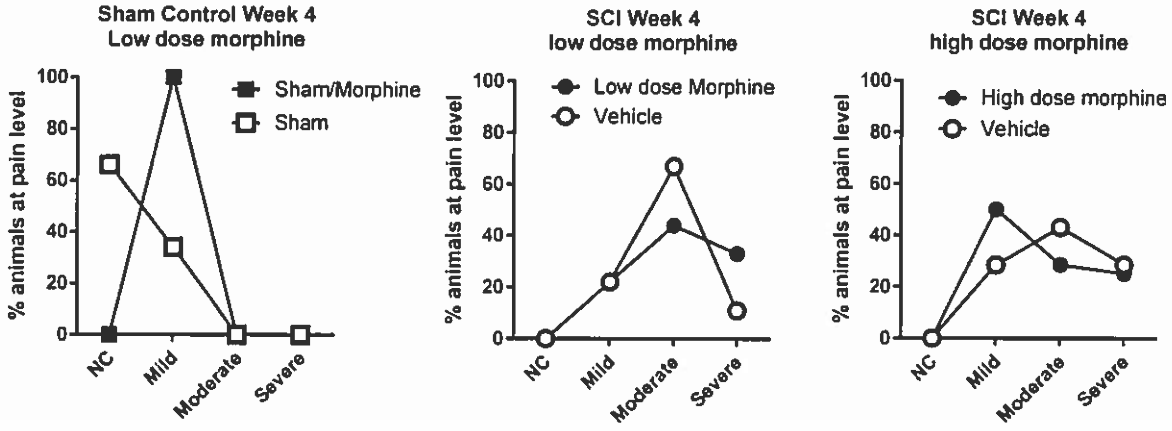


Figure 9.



- **Stated goals not met.** Nothing to report since last year.

- **Training and personal development opportunities.** Nothing to report.

- **Dissemination to communities of interest.** Nothing to report.

- **Future goals.** In the next quarter we plan to complete RT-PCR and flow cytometry analyses in the low and high morphine exposure mice. At the same time we will commence experiments outlined in Subtask 4A to determine the impact of ethanol exposure.

6. IMPACT

c. **Impact on the fields of cannabinoids and spinal cord injury.** Our results demonstrate that treatment with CBD can attenuate the development of pain sensitivity following SCI in a mouse model. These results provide the first evidence for a protective effect of CBD in an animal model of neuropathic pain following spinal cord injury and support ours and other's findings in other animal models of neuropathic pain. The results from these experiments also heavily implicates the ability of CBD to suppress T cell activation and recruitment in its protective action against development of central neuropathic pain. Our results also demonstrate that in SCI, chronic morphine exposure leads to less thermal hypersensitivity than it does in sham treated mice, but that lower concentrations of morphine are associated with more hyperalgesia in SCI mice than higher concentrations of morphine.

d. **Impact on other fields.** These discoveries have implications for drug discovery in that CBD may be highlighted as a novel chemical scaffold for a protective molecule against immune cell activation and CNS infiltration. These discoveries have implications for the understanding of the mechanisms of CBD in other disease models with a significant immune/inflammatory component.

e. **Impact on technology transfer.** These results are likely to make an impact on commercial technology as the investigators are currently in communication with several biotechnology companies regarding patent concepts for CBD and/or synthesis of CBD analogues for distinct therapeutic targets.

f. **Impact on society.** Results such as the ones presented here wherein non-psychoactive cannabinoid agents show evidence-based efficacy in models of chronic pain improve public knowledge and attitudes on the therapeutic potential of cannabinoid-based treatment strategies.

7. CHANGES/PROBLEMS

g. **Changes in approach.** Nothing to report.

h. **Delays in action.** The need to explore multiple concentrations of morphine osmotic pump exposure delayed progress. We will rectify this by commencing with the alcohol studies at the same time that we finish cellular and molecular assessments in the morphine studies.

i. **Changes in expenditures.** Nothing to report.

j. **Changes in use or care of human subjects, vertebrate animals or select agents.** Nothing to report.

8. PRODUCTS

k. **Publications, conference papers, and presentations.** Oral presentation by project-supported Co-I Ronald Tuma, PhD, at the annual International Cannabinoid Research Symposium in Bukovina Poland in June 2016 entitled "Targeting spinal cord injury-associated neuropathic pain with Cannabidiol."

l. **Websites.** Nothing to report

m. **Technologies/techniques.** Nothing to report

n. **Inventions, patent applications, licenses.** Nothing to report

o. **Other products.** Nothing to report

9. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

p. **Personnel**

- **Name: Sara Jane Ward**
  Project Role: PI
  Person months worked: 3
  Contribution to project: Supervising the research program, designing the experiments, provide assistance in the laboratory when needed, analyzing the data (together with the other members of the research team), and composition of the reports and manuscripts.
- **Name: Ronald Tuma**
  Project Role: Co-investigator
  Person months worked: 2.8
  Contribution to project: Supervising the procedures directly related to spinal cord injury in mice and assisting the PI in design of experiments and interpretation of results.
- **Name: Doina Ganea**
  Project Role: Co-investigator
  Person months worked: 1.2
  Contribution to project: Supervising personnel regarding the immunology evaluations and assisting Dr. Ward in the planning of the experiments and analysis of data related to these endpoints.
- **Name: Hongbo Li**
  Project Role: Postdoctoral Fellow
  Person months worked: 9
  Contribution to project: Conducting the spinal cord injuries and behavioral assessments, as well as immunohistochemistry and analysis of data.
- **Name: Weimin Kong**
  Project Role: Research Associate
  Person months worked: 6
  Contribution to project: Conducting flow cytometry, RT-PCR, and cell culture analyses.

q. **Changes in active other support**

- **Sara Jane Ward.** Nothing to report
- **Ronald Tuma.** Nothing to report
- **Doina Ganea.** Nothing to report

r. **Partner organizations.** Nothing to report.

10. SPECIAL REPORTING REQUIREMENTS

s. **Collaborative award.** Nothing to report

t. Quad Chart. Attached.

Modulation of invading and resident inflammatory cell activation as a novel way to mitigate spinal cord injury-associated neuropathic pain

Log Number MR130262

PI: Sara Jane Ward, PhD **Org:** Temple University School of Medicine

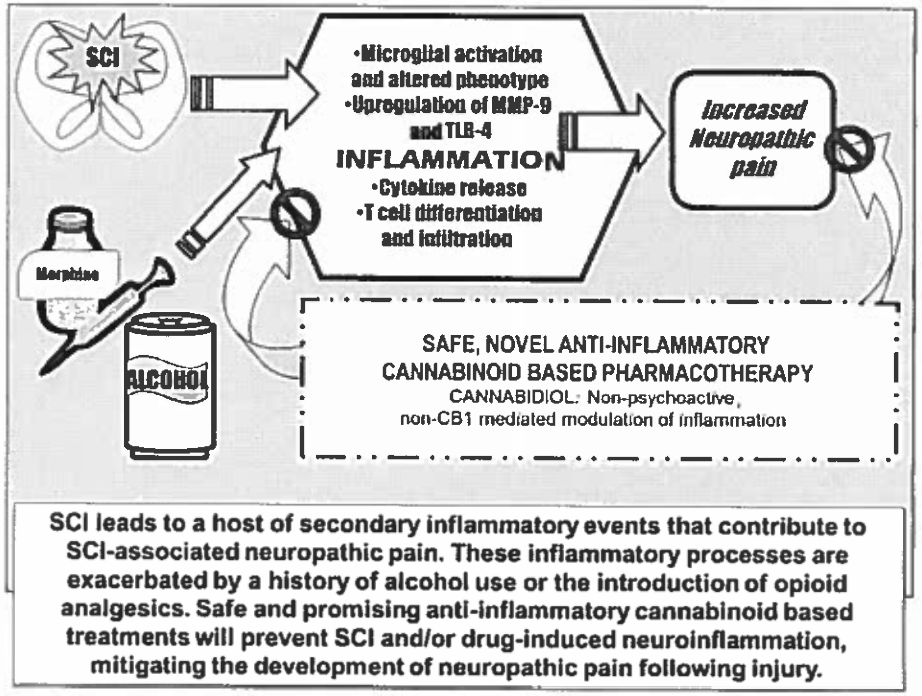
Study Aims

Aim 1: Determine the effect of CBD on the development and maintenance of spinal cord injury neuropathic pain (SCI-NP) and associated inflammatory markers, including1) the M1 microglial phenotype, and 2) IL-17 producing T (Th17) cells.

Aim 2: Determine the effect of opioid exposure alone and in combination with SCI on the same inflammatory and neuropathic endpoints, as well as the ameliorative effects of CBD. **Aim 3** will determine the effect of chronic alcohol consumption alone and in combination with SCI on the same inflammatory and neuropathic endpoints, as well as the ameliorative effects of CBD.

Approach: The strengths of our approach include the following:

- Identification of non-neuronal inflammatory mechanisms underlying SCI-NP, such as M1 microglial phenotype and Th17 T cell invasion.
- Examination of how the pro-inflammatory effects of opioid or alcohol exposure exacerbate development of neuropathic pain following SCI.
- Targeting CBD as a cannabinoid-based pharmacotherapy. Strengths of this approach include 1) its non-psychoactive pharmacological profile as it does not activate CB1 receptors, 2) the wealth of safety data already available from human studies of other disease endpoints, 3) evidence that it is an effective anti-neuropathic agent as a component of the medication Sativex, and 4) intense interest already shown by the FDA in the determination of its full range of therapeutic benefits.



SCI leads to a host of secondary inflammatory events that contribute to SCI-associated neuropathic pain. These inflammatory processes are exacerbated by a history of alcohol use or the introduction of opioid analgesics. Safe and promising anti-inflammatory cannabinoid based treatments will prevent SCI and/or drug-induced neuroinflammation, mitigating the development of neuropathic pain following injury.

Timeline and Cost

Activities	14 /15	15/16	16/17
Aim 1 Effect of CBD on SCI-NP development and changes in novel immune targets	■		
Aim 2 Opioid/SCI-NP interactions on immune targets and effect of CBD		■	
Aim 3 Alcohol/SCI-NP interactions on immune targets and effect of CBD			■
Estimated Direct Costs ($K)	**$250**	**$250**	**$250**

Goals/Milestones

CY14 Goals

- ❑ Determine effects of CBD treatment on the development of SCI-NP and associated inflammatory markers
- ❑ Determine the time course of glial and immune cell expression and phenotype during SCI-NP and modulation by CBD

CY15 Goals

- ❑ Quantify markers following opioid exposure
- ❑ Investigate combined opioid/SCI effects on inflammation and neuropathic pain
- ❑ Test non-psychoactive cannabidiol on pain/inflammatory outcomes

CY16 Goals

- ❑ Quantify markers following alcohol consumption
- ❑ Investigate combined alcohol/SCI effects on inflammation and neuropathic pain
- ❑ Test non-psychoactive cannabidiol on pain/inflammatory outcomes